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Prophylaxis against chemical warfare agents may reduce the extent of the intoxication and thereby improve the prognosis for the patient. Since treatment for intoxications with organophosphorous (OP) acetylcholinesterase (AChE) inhibitors is still far from ideal, research efforts are devoted towards finding an effective prophylaxis. A successful prophylaxis should fulfill 3 conditions: 1) offering a high protection rate, 2) causing no side effects, and 3) protecting against post-intoxication incapacitation. The mechanism of action of the currently available prophylaxis pyridostigmine is to protect a fraction of the enzyme from binding with irreversible OP AChEinhibitors. However, because if its structure, pyridostigmine hardly penetrates the brain and will therefore not protect the brain AChE sufficiently. This may lead to brain damage and post-intoxication incapacitation. Therefore, alternatives for pyridostigmine should be evaluated. For the near future, prophylaxis with physostigmine and scopolamine seems to be the best choice and most promising alternative to prevent OP intoxication. For the intermediary term, research towards compounds with another mechanism of action, like procyclidine, should be considered. For the long term the use of scavengers can be evaluated. This report is a new reviewed version of the former report PML 2002-A94 "Profylaxis against Organophosphorous nerve agents – state of the art".

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Managementuittreksel

TNO | Kennis voor zaken

Profylaxe tegen organofosfaat zenuwgassen - stand van zaken

Probleemstelling

In opdracht van het Ministerie van Defensie is een overzicht gemaakt door TNO Defensie en Veiligheid over de stand van zaken binnen het onderzoek naar profylaxe (voorbehoeding) tegen agentia (ziekteverwekkende stoffen) in chemische wapens. Aangezien uitsluitend profylactische middelen tegen organofosfaat zenuwgassen bestaan, beperkt dit rapport zich hiertoe.

Momenteel wordt pyridostigmine, een carbamaat, gebruikt als profylaxe.

De werking van pyridostigmine berust op de reversibele binding met het target enzym cholinesterase, waardoor een deel van het enzym wordt afgeschermd. Hierdoor kan het zenuwgas het enzym niet binden. Door de reversibele binding van pyridostigmine komt het enzym weer vrij. De dosis pyridostigmine is zo gekozen dat de hoeveelheid enzym dat per tijdseenheid spontaan weer los komt voldoende is om vitale levensfuncties te kunnen waarborgen. Echter, pyridostigmine dringt slecht door de bloed-hersenbarrière waardoor het nauwelijks beschermt tegen centrale effecten, waaronder post-intoxicatie incapacitering. Mede om deze reden wordt er onderzoek verricht naar alternatieven voor de vervanging van pyridostigmine. Dit overzicht over de stand van zaken binnen het profylaxe-onderzoek is nodig voor de besluitvorming omtrent de vervanging van de huidige profylaxe door een effectievere en betere voorbehandeling. Dit rapport betreft een nieuwe herziene versie van het eerder uitgebrachte rapport PML 2002-A94 "Profylaxis against Organophosphorus nerve agents – state of the art".

Beschrijving van de werkzaamheden

Het onderzoek naar de stand van zaken omtrent de profylaxe is gebaseerd op onderzoeksgegevens van de laatste 15 jaar binnen TNO Defensie en Veiligheid en op gegevens uit artikelen en studies uit open bronnen.

Toepasbaarheid

Om zich te beschermen tegen de schadelijke inwerking van organofosfaten kan men preventief geneesmiddelen toedienen. De werking van een voorbehandeling berust op het feit dat de neurotransmissie binnen het cholinerge transmissiesysteem normaal of voldoende kan blijven functioneren. Dit kan bereikt worden door een deel van het target enzym af te schermen voor organofosfaat¬verbindingen, extra enzym toe te voegen of te compenseren binnen andere mechanismen, zoals de afgifteremming van transmitterstof, of regulatie binnen andere transmitter¬systemen. Van belang bij profylaxe is dat het niet leidt tot ongewenste neveneffecten maar wel beschermt tegen sterfte en post-intoxicatie incapacitering. Behandelingen die voldoen aan deze drie

voorwaarden kunnen worden overwogen als alternatief voor de huidige profylaxe.

Vervolgafspraken

Deze studie maakt deel uit van een breder programma, te weten de passieve verdediging tegen NBC wapens (V013). In juni 2005 is een advies uitgebracht voor de vervanging van pyridostigmine door fysostigmine. Andere alternatieven zullen op de langere termijn verder onderzocht kunnen worden in het vervolgprogramma V502.

PROGRAMMA	**PROJECT**
Programmabegeleider Drs. A.S. de Koning, MGFB	Projectbegeleider Mw M.W. Valstar, Apotheker, CDC/MGFB
Programmaleider Dr. ir. M.S. Nieuwenhuizen, BU Biologische en chemische bescherming	Projectleider Dr. I.H.C.H.M. Philippens, BU Biologische en chemische bescherming
Programmatitel NBC-Bescherming	Projecttitel 208 Profylaxe Stand van Zaken CW
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Summary

Prophylaxis against chemical warfare agents may reduce the extent of the intoxication and thereby improve the prognosis for the patient. Prophylaxis is only available for nerve agents. Exposure to nerve agents is currently not restricted to the battlefield. The problem of destruction of the chemical stockpiles after the Chemical Weapons Convention and the abuse by terrorist organizations will increase the risk of exposure. Since treatment for intoxications with at least some of these organophosphorous (OP) acetylcholinesterase (AChE) inhibitors is still far from ideal, research efforts are devoted towards finding an effective prophylaxis.

A successful prophylaxis optimally should fulfil 3 conditions: 1) it should offer a high protection rate, 2) it should not cause side effects, and 3) it should protect against post-intoxication incapacitation.

The mechanism of action of the currently available prophylaxis pyridostigmine, a reversible AChE inhibitor, is to protect a fraction of the enzyme from binding with irreversible OP AChE-inhibitors. However, because of its structure, pyridostigmine hardly penetrates the brain and will therefore not protect the brain AChE sufficiently. This may lead to brain damage and post-intoxication incapacitation. Therefore, alternatives for pyridostigmine should be evaluated.

For the near future, subchronic prophylaxis with physostigmine and scopolamine seems to be the best choice and most promising alternative to prevent OP intoxication. For the intermediary term, research towards compounds with another mechanism of action, like procyclidine, should be considered. And for the long term the use of scavengers can be evaluated.

This report is a new reviewed version of the former TNO-report PML 2002-A94 'Profylaxis against Organophosphorus nerve agents – state of the art'. The latest information from the literature is inserted in this new version.

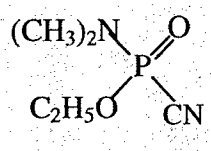
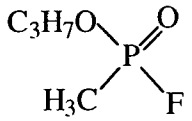
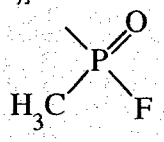
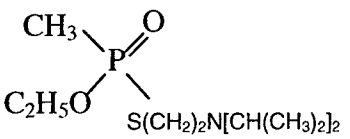
Contents

1 Introduction

1.1 Nerve agents

A chemische wapens agent is a chemical compound that is used in military operations or by terrorist organisations to incapacitate, seriously injure or kill personnel through its chemical properties. Nerve agents or organophosphates (OPs) belong to these CW agents. In Table 1 the structural formulas of most well known CW agents are shown. The first nerve gas was tabun (Ethyl N-methyl-phosphoramido cyanidate) (GA), followed by sarin (Isopropyl methyl phosphonofluoridate) (GB) and soman (Pinacolyl methylphosphonofluoridate) (GD). These agents are called the G-agents and were developed for the German army shortly before and during World War II.

Table 1 CW agents and their structural formula.

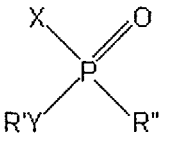
Common and chemical names	Structural formula
Tabun (GA) Ethyl N-dimethyl-phosphoramido cyanidate	
Sarin (GB) Isopropyl methyl phosphono-fluoridate	
Soman (GD) Pinacolyl methylphosphono-Fluoridate	$(CH_3)_3C[CH(CH3)]O$ 
VX Ethyl S-2-diisopropyl-aminoethyl methyl-phos-phorothiolate	

Later on, in 1952, the fourth agent VX (Ethyl S-2-diisopropylaminoethyl methyl-phosphorothiolate) (V-agent) was developed by the collaborative action of laboratories in the United Kingdom, the United States, and Canada. In recent years other nerve agents, like cyclohexyl sarin (GF) or 2-dimethylaminoethyl-(dimethylamido)-phosphono fluoridate (GV) were synthesised. Due to the existence of these nerve agents and the likely development of new nerve agents, defence organisations are forced to search for adequate protection of their soldiers. Therefore, a lot of research has been carried out since World War II, on the physical (protective gear / gasmask) and medical (prophylactic and therapeutic measures) protection.

Now, in the beginning of the 21st century, that the Chemical Weapons Convention has been ratified by almost all countries, the threat of exposure to chemical agents should be minimised. Why still searching for a successful prophylaxis against nerve gas intoxication? Will there still be the risk of intoxication by a nerve gas? Unfortunately the answer has to be yes. Preparation of nerve gases is relatively simple. A small amount may already be lethal. Terrorist groups can easily manufacture this kind of weapon when all basic substances are available. An example was the terrorist attack with the nerve gas sarin in the Tokyo underground in 1995. The sectarian group, responsible for this attack, had also used VX at another, earlier, occasion. The best cure is preventing the risk of exposure. This may be achieved by the Chemical Weapons Convention that went into force in April 1997. However, in situations of war with countries that did not ratify the convention or terrorist actions the CWC is not a preventive measure. Furthermore, after ratification of the Chemical Weapons Convention the problem of destruction of these weapons has arisen. Worldwide one may expect large nerve gas stockpiles to be present, particularly in the former Soviet Union and the United States. Destruction of these weapons can only be carried out when sufficient protective measures have been taken; one of them being a protective prophylaxis against intoxication.

1.2 Mechanism of action of organophosphates

Nerve agents are derivatives of phosphoric acid, all with the general formula (Schrader, 1952):



Y = O, S
X = F, CN, N3, S(CH2)2S+R2", S(CH2)nNR2"
R' = alkyl, cycloalkyl or H
R" = alkyl, dialkylamino

The nerve agents are alkyl esters of either a dialkylaminocyanophosphoric acid (tabun), alkylfluorophosphoric acids (sarin and soman), or an S-dialkylaminoethyl alkyl-phosphonothiolic acid (VX). Toxicologically these agents are in many aspects similar to many of the commercially available organophosphorous pesticides, such as parathion, TEPP or tetram and carbamates.
The physiological action of all OPs results from their inhibition of the enzyme acetylcholinesterase (AChE). AChE is necessary for the communication between nerve cells and effector organs and for the function of muscles and more complex behaviour, such as learning and memory. AChE is responsible for the hydrolysis of the ester acetylcholine (ACh, see Figure 1). ACh serves as a neurotransmitter in the central nervous system (CNS) and at peripheral neuromuscular junctions.

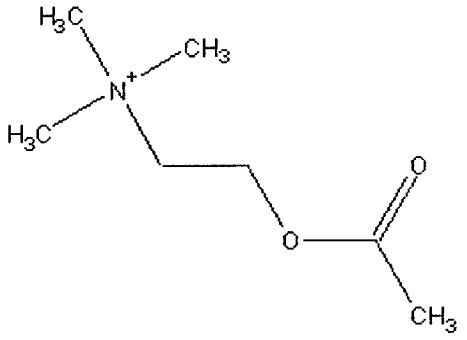


Figure 1 Structural formula of ACh.

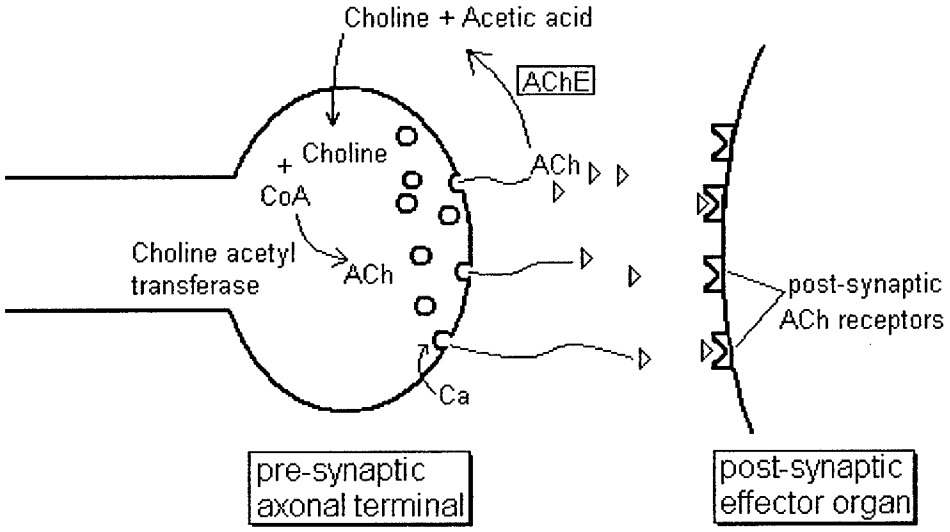


Figure 2 Schematic representation of the cholinergic synapse illustrating the different steps in acetylcholine mediated neurotransmission.

Figure 2 shows the cholinergic synapse illustrating the different steps leading to ACh mediated neurotransmission. Released ACh diffuses across the synaptic or junctional cleft. At the postjunctional membrane it reacts with specialized receptor sites resulting in an increase of the ionic permeability, or conductance, of the membrane. To serve as a neurotransmitter, ACh must be removed or inactivated immediately after activation of the post-synaptic receptor. AChE is responsible for the hydrolysis of ACh to choline and acetic acid. The active site of AChE is situated in the centre of the enzyme at the bottom of a deep and narrow gorge. Binding of the quaternary ammonium ion of ACh takes place to the 'electron cloud' of the 14 aromatic residues that are situated in the gorge (Sussman *et al.*, 1991; Ripoll *et al.*, 1993). Choline will be taken up by the pre-synaptic neuron from the extra-cellular fluid by active transport and can be used again for de novo synthesis of ACh.

Organophosphorus compounds possess a P=O group that allows the same reaction to occur with AChE as between AChE and the C=O group of ACh. However, instead of an acetylated enzyme, now a phosphorylated enzyme is formed. When, in case of, the phosphorylated enzyme a group (X) leaves the enzyme leaving a phosphorous behind in

the AChE then the binding has become covalent and the enzyme is inactivated. Depending on the type of OP that has bound onto the enzyme, the enzyme can reactivate by hydrolysis. This reactivation occurs very slowly but can be reinforced by an oxime. When in case of the phosphorylated enzyme an alkyl group (R') leaves the enzyme then the enzyme can not be reactivated and is now irreversibly changed; this reaction is called the ageing reaction (Hobbiger, 1956; Berends *et al.*, 1959; Fleisher and Harris, 1965).

1.3 Symptoms of organophosphate intoxication

The intoxication effects after OP exposure are mainly caused by the accumulation of ACh leading to the following symptoms (Taylor, 1996):

Peripheral muscarinergic:	pupil narrowing (miosis), extensive salivation, bronchospasm, and intestinal spasm.
Peripheral nicotinergic:	tremors, muscle fasciculations and tetanic muscle contractions leading to paralysis through the absence of neuromuscular transmission.
Central:	extreme confusion, convulsions, unconsciousness, and/or paralysis of the respiratory centre that eventually can lead to death.

The early signs of OP intoxication are confusion, blurred vision due to miosis (pupil narrowing), and tightness of the chest. A severe OP intoxication leading to an AChE inhibition of 95% or more will be lethal through respiratory failure, i.e. laryngospasm, bronchoconstriction, increased tracheobronchial and salivary secretion, and peripheral and central respiratory paralysis. A near lethal dose will cause irreversible brain damage in the cholinergic areas such as the hippocampus. This can lead to concentration, memory, and learning disabilities. Another effect of these anti-AChE agents can be a cholinomimetic action of the muscarinic type at autonomic effector organs.

1.4 Current treatment regime of organophosphate intoxication

Adequate protection against exposure to OPs can be achieved by wearing protective gear and a gas mask. In case a soldier is not sufficiently protected he might be exposed and therapeutic measures should be taken. The cardinal principles of therapy for OP intoxication are enzyme reactivation with an oxime, parasympathetic blockage (with atropine), anticonvulsant, decontamination, and ventilation. As quickly as possible, the contaminated skin area should be decontaminated with a decontaminant. These compounds inactivate the nerve agent before it can enter the body. Examples are RSDL (Reactive Skin Decontamination Lotion) and hypochlorite. An additional aspect of decontamination is physical removal from the skin. When a nerve agent has entered the body, therapy should be aimed at reactivation of AChE before the ageing reaction has occurred (Berends, 1964; Fleisher, 1965). Hydrolytic regeneration of acetylated AChE can be obtained with an oxime (Wilson, 1954). Wilson found that this could be obtained by a molecule containing both a quaternary N-atom and an oxime group, spaced at an appropriate distance, these compounds are called oximes. When ageing of AChE has occurred no reactivation of AChE can be obtained. Aging occur after exposure to soman very rapidly within a few minutes. In such a case therapy with atropine is possible. Atropine prevents the actions of the accumulated acetylcholine by blocking the muscarinic ACh receptor (Koplovitz, 1995). This antagonistic effect of atropine was already described by Kleinwaechter (1864). Not only due to the rapid ageing of the

phosphylated AChE and the poor reactivatability of non-aged enzyme (oxime-resistancy), but also the predominant effects on the CNS, and the persistence in 'depots' in the body make the nerve agent soman one of the most dangerous and difficult to treat OP intoxication (Wolthuis, 1981). For these reasons prophylaxis has been considered in addition to treatment.

1.5 Prerequisites for a successful prophylaxis

A successful prophylaxis to be used against OP intoxication optimally should fulfil 3 conditions:

1 it should offer a high protection rate;
2 it should not cause side effects, and;
3 it should protect against post-intoxication incapacitation.

Protection against lethality

The current prophylaxis against OP intoxication is aimed at protecting a fraction of the AChE from irreversible binding by the OP, thereby allowing sufficient active AChE to be present in the body. Therefore, successful prophylaxis can be achieved by compounds that bind to AChE and spontaneously hydrolyses from the enzyme. Commonly such compounds are called reversible inhibitors. This is a seemingly paradoxical situation of 2 compounds with the same mechanism of toxicity in which one protects against the toxicity of the other. However, due to the reversible binding of the prophylaxis compound, like carbamates such as pyridostigmine and physostigmine, with AChE, AChE activity may return fast enough to prevent lethality following OP intoxication (Berry and Davies, 1970; Dirnhuber *et al.*, 1979; Gordon *et al.*, 1978; Harris *et al.*, 1980). This strategy should offer a high protective ratio expressed as the LD_{50} of OP in pretreated animals divided by LD_{50} of OP in unpretreated animals. Compounds that show reversible binding to AChE are carbamates (currently used) and reversible OP inhibitors like the insecticide tetraethyl pyrophosphate (TEPP) (Clermont, 1854) or phosphoramidates. At present, no phosphoramidate with a high protective ratio has been identified (Philippens *et al.*, 1996; Melchers *et al.*, 1994; Langenberg *et al.*, 1996). The drawback of TEPP was the necessity of an oxime to reactivate the enzyme after intoxication.

Side effects

When given to healthy persons side effects of drugs ought to be minimized as much as possible; in particular when such prophylaxis is required for a longer period of time. Since prophylaxis against OP intoxication should also inhibit AChE in the brain, the accumulation of ACh could lead to unwanted side effects like reaction, concentration, memory, and learning disabilities. The side effects can be counteracted by a cholinolytic that binds to the post-synaptic receptor without affecting it whilst preventing the binding of the transmitter ACh. The cholinolytics used are: atropine sulphate and scopolamine (Koplovitz, 1995; Leadbeater *et al.*, 1985). These drugs have proven to be able to counteract effects on autonomic effector cells and on cortical and subcortical sites in the CNS, where the receptors are largely of the muscarinic type (Berry and Davies, 1970).

Post-intoxication incapacitation

Most regimens are effective in preventing lethality from OP intoxication but do not prevent toxic effects and incapacitation. Incapacitation due to the over-stimulation of the peripheral ACh receptors leads to symptoms such as abdominal cramps, a decrease

in heart rate, hypersalivation, urinary incontinence, muscle weakness, fasciculation, diarrhoea, and blurred vision. Incapacitating effects resulting from over-stimulation of central ACh receptors lead to convulsions. Severe convulsions induce irreversible brain damage in cholinergic areas. In case chemical weapons are used during wartime, exposure to the nerve agent around the centre of an explosion will almost certainly be lethal when no adequate protection is present. However, the nerve gas will not only contaminate the area of the explosion but also the surroundings. In the surroundings the concentration of the nerve gas will be lower and, therefore, only moderate to light intoxication might occur. This certainly forms a risk factor. Due to this type of exposure in the contaminated area people may suffer from post-intoxication incapacitation.

1.6 Current prophylaxis

Currently pyridostigmine bromide (Figure 3) is used in most NATO countries as a prophylaxis against OP intoxication in combination with a therapy consisting of an oxime, atropine, and an anticonvulsant (diazepam). In the past, pyridostigmine has also been in use clinically in the treatment of glaucoma and for many years in the treatment of myasthenia gravis.

Protection against lethality
Pyridostigmine is a carbamate that binds to the enzyme AChE in a reversible manner (Watts and Wilkinson, 1977). In Table 2 it is shown that pyridostigmine in combination with a post intoxication therapy protects effectively against lethality in a number of species (Gordon, 1978; Leadbeater, 1985; Dirnhuber *et al.*, 1979). However, pyridostigmine alone doesn't protect against the toxicant (Gordon *et al.*, 1978).

Table 2 Protective ratios against OP intoxications after prophylactic treatment with pyridostigmine (PYR) combined with post-intoxication therapy in different animal species.

Species	Dose PYR (mg/kg)	Prophylactic interval (min)		Protective ratio			
				tabun	Sarin	soman	VX
Rat	0.075 (im)	20	+	1.2	1.5	1.7	5.0
Rabbit	0.1 (im)	30	+	4.6	27.0	2.7	5.0
Guinea pig	0.1 (im)	30	+	22.0	21.5	5.3	17.9
Guinea pig	0.1 (im)	30	*	17.0	3.8	5.5	2.9
Marmoset	0.2 (iv)	10	**			15.0	
Rhesus	0.2 (iv)	15	**			28.0	

Protective ratio: LD_{50} of OP in pretreated animals / LD_{50} of OP in non-pretreated animals.
\+ oxime P2S (30 mg/kg) and atropine sulphate (AS) (17.4 mg/kg) (im) were given therapeutically 1 min after OP intoxication.
* Atropine sulphate (17.4 mg/kg) (im) was given therapeutically 1 min after OP intoxication.
** Atropine sulphate (4 mg/kg) (im) was given therapeutically 1 min after OP intoxication.
(data collected from Gordon *et al.*, 1978 and Dirnhuber *et al.*, 1979).

Side effects

During Operation Desert Storm soldiers were given pyridostigmine in a blister pack containing twenty-one 30-mg pyridostigmine tablets. Each soldier received instructions to take one tablet every 8 hours. This dose of pyridostigmine was expected not to show undesirable cholinergic effects. Nevertheless, peripheral and central side effects were recorded (Keeler *et al.*, 1991). About 50% of the pretreated personnel noted not incapacitating physiologic changes, like increased flatus, abdominal cramps, soft stools, and urinary urgency. In 1% of the pretreated personnel, symptoms were causing unacceptable discomfort. 0.1% of the personnel discontinued the prophylaxis on medical advice. Even after several years some symptoms, like joint pain, fatigue, weight change, and sleep disturbances, were still present, according to some authors (Stuart *et al.*, 2002). However, under laboratory conditions fewer effects were found (Gawron *et al.*, 1990; Cook *et al.*, 2001). Furthermore, the effects were considered to be unrelated to cholinesterase inhibition (Cook *et al.*, 2001). This implies that symptoms may be exacerbated by the unavoidable physiological and psychological stresses of war. First of all stress itself could be an important factor, however, stress also enhances the passage of pyridostigmine across the blood-brain barrier (Friedman, 1996). In the Operation Desert Storm nine cases of pyridostigmine self-poisoning were encountered. These individuals only suffered from peripheral cholinergic symptoms such as abdominal cramps, diarrhea, hypersalivation, blurred vision etc, whereas no effects on the central nervous system were observed (Almog, 1991). These self-poisonings do not indicate that any so-called 'Gulf War Syndrome' could be caused by pyridostigmine.

Post-intoxication incapacitation

The structure of pyridostigmine contains a quaternary nitrogen atom (see Figure3). For this reason pyridostigmine hardly penetrates the brain and will not inhibit brain-AChE. A single im injection of pyridostigmine (131 μg/kg) caused 58.5% inhibition of blood AChE and no inhibition of brain AChE, while a tertiary derivative 3-(N,N-dimethylcarbamyloxy)-1-methyl-Δ3-tetrahydropyridine (THP) caused, given at a comparable dose, 30.0% inhibition of blood AChE and 25.3% inhibition of brain AChE (Ray, 1991). This is in accordance with findings in behavioural studies: no central effects were found after pyridostigmine administration compared with low dose levels of physostigmine or soman (Wolthuis *et al.*, 1995). Therefore, pyridostigmine does not protect the CNS against the toxic influences of OPs.

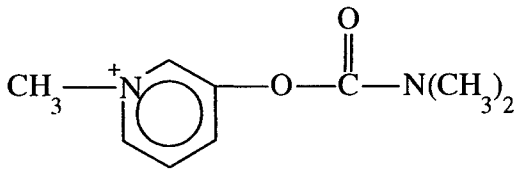


Figure 3 Structural formula of pyridostigmine.

2 Extrapolation of prophylaxis protocols in animals to man

Obviously, it is impossible to test the efficacy of a prophylaxis protocol against an OP challenge in human volunteers. Therefore, studies with experimental animals are inevitable. This is also the case in studies towards alternatives for the current prophylaxis with pyridostigmine. Therefore, the question about the application of experimental data in animals in man may arise.
In the past, research with anti-ChE was performed in the rat (Wolthuis and Vanwersch, 1984). In contrast to man, rats have high amounts of carboxylesterase in their blood. These esterases can act as scavengers for the anti-ChE drugs. For this reason the rat is not a suitable model for man. Therefore, animals that have relatively low blood carboxylesterase levels are preferable models, i.e., guinea pig (Maxwell *et al.*, 1987) and marmoset monkey. The marmoset also proved to be a good model for man to measure enzyme reactivity after soman intoxication (Van Helden *et al.,* 1983). Also concerning the decarbamoylation rates of carbamate-inhibited plasma and red cell cholinesterases the non-human primates are the best animal model for extrapolation from these animal studies to man (Wetherell and French, 1991). Furthermore, toxicokinetic studies reported that the concentration time profile of soman in guinea pigs resembles that of the marmoset monkey more closely than that of the rat (Benschop and De Jong, 1991). For these reasons the guinea pig and the marmoset monkey have been used for experimental work carried out at TNO.

Knowing that behaviour is regulated by the nervous system, effects of chemicals on the function of the nervous system, e.g., blockade of receptors, release of transmitters, etc., will probably affect behaviour as the consequence of the disturbed systems. In case of OP intoxication some examples are present in humans. By observing the symptomatology in man, animal models can be created that develop comparable signs, although some symptoms like hallucinations or headache are difficult to detect in animal models. On the other hand, behavioural models measuring deficits in motor, sensory and cognitive functions reveal qualitative effects that have some predictive value when compared with similar effects in humans. However, it is difficult to predict which functional domain in the brain is affected. Therefore, development of a variety of read-out systems is needed. Still the question exists whether the data obtained from the different read-out systems for the guinea pigs and the marmoset monkeys are predictive for man. Therefore, an approach is needed to extrapolate from experimental data in the guinea pig and the marmoset to man. As stated before, the marmoset has been shown to be a suitable model for man in OP toxicity studies (Van Helden *et al.*, 1983). Likewise, from a toxicokinetic point of view the guinea pig appeared to be a better model for the marmoset than the rat (Benschop and De Jong, 1991). However, since marmosets or guinea pigs are not miniature humans, extrapolation on the basis of body weight, metabolic rate or ventilation rate will not be adequate. For exposure studies an exponent of the LD_{50} value is mostly used. For carbamate (prophylaxis) dosages the level of AChE inhibition is used. However, the most suitable approach for interspecies extrapolation is via physiologically based pharmacokinetic modelling (PBPK) (Dedrick *et al.*, 1973; King *et al.*, 1983; Ramsey and Andersen, 1984; Lutz *et al.,* 1984), albeit that in the case of soman one should replace 'pharmacokinetic' with 'toxicokinetic'. PBPK models represent the mammalian system in terms of specific tissues or groups of tissues, connected by arterial and venous blood flow pathways. They consist of a set of differential equations describing the mass-balance in the various tissues and groups of tissues. Based on these differential equations, time-dependent toxicokinetic data can be

simulated. The model contains physiological parameters, such as tissue volumes and blood flow rates, and parameters specific for the chemical agent under investigation, such as tissue/blood partition coefficients and metabolic parameters. The coherent relationship among anatomical and physiological characteristics of different species provides the basis for cross-species scaling of toxicokinetic data described in such a model and extrapolation eventually to man. Nowadays, PBPK models are used extensively for risk assessment purposes. The results of this type of modelling are accepted by regulatory organizations.
Langenberg *et al.* (1997) have reported a PBPK model for the intravenous toxicokinetics of soman in the atropinized guinea pig. This model was validated by comparing the predictions of the model with the actual toxicokinetic data, i.e., measured time courses of arterial blood levels of soman following iv bolus administration of doses of soman corresponding with 0.8, 2 and 6 x LD_{50}. The model also contains differential equations for simulation of carbamate prophylaxis. Furthermore, AChE activities can be predicted at any point in time during the simulation in all compartments defined in the model. Presently, the physiologically based model developed by dr Langenberg (TNO) for the intravenous toxicokinetics of soman in the atropinized guinea pig is being adapted to allow modelling of nose-only exposure. Concomitantly, a model for the marmoset is under development. Both projects are part of R&D programme V013. When these models are adequately validated, a model for the toxicokinetics of soman in man will be constructed. Until these models have been fully validated it remains uncertain whether our findings in the guinea pig and marmoset monkey can be directly extrapolated to man.

3 Alternatives for pyridostigmine as a prophylaxis

For being an effective prophylaxis a compound should meet the earlier mentioned three conditions: it should offer a high protection rate, not cause any side effects, and protect against post-intoxication incapacitation. To find a new and better prophylaxis against OP intoxication different options were mentioned. One of these is the carbamate physostigmine. There were indications that this carbamate was more effective than pyridostigmine. However, this carbamate which easily penetrates into the brain may cause undesired side effects. Another alternative is the phosphoramidate compounds. These are also OPs but with a reversible way of action and relatively few side effects. These compounds seem to be very promising depending on the route of administration. Unfortunately, the most practical route of administration (orally) did not lead to a high protection rate, probably due to unfavourable pharmacokinetics. A different approach is the addition of stochioimetric or catalytic scavengers in the blood. These can be human butyrylcholinesterases. Because this enzyme already exists in the body, side effects will be minimal. For the use as a prophylaxis a constant level of this enzyme is necessary, which may be difficult. This type of treatment is already in use against cocaine intoxication. And finally, new insights will lead to new compounds and new way of actions in the prophylaxis against OPs.

For the short term replacement of pyridostigmine by physostigmine seems to be the most promising alternative. For the near future other alternatives like phosphoramidates or drugs with another mechanism of action will be tested. And for the longer term the use of scavengers (presumably catalytic) will be evaluated.

3.1 Phosphoramidates as an alternative for pyridostigmine

In the late nineteen eighties and early nineteen nineties p-nitrophenyl phosphoramidates were studied as candidate prophylaxis compounds against soman. These phosphoramidates are potent inhibitors of acetylcholinesterase, but the inhibited enzyme reactivates spontaneously at a rate that depends on the chemical structure of the compound (Langenberg *et al.*, 1988). Intravenous prophylaxis of guinea pigs with p-nitrophenyl phosphoramidates followed by a subcutaneous challenge with soman and intraperitoneal administration of atropine protected the animals to a large extent from mortality (Langenberg *et al.*, 1996). The most promising compound was ethyl p-nitrophenyl phosphoramidate, which offered a protective ratio (PR) against soman of 4.9, whereas under the same experimental conditions the PR for pyridostigmine was only 2.0. Furthermore, the phosphoramidate-pretreated animals that survived the soman challenge were in a remarkably good condition. Next, it was attempted to administer ethyl p-nitrophenyl phosphoramidate via a more practical route, i.e., subcutaneously and orally. Unfortunately, for both administration routes the PR of this compound was disappointingly low, i.e. insignificant (Melchers *et al.*, 1994, Langenberg *et al.*, 1992). It was concluded that this was due to suboptimal pharmacokinetic properties of the compound like water solubility, partition coefficient and stability towards hydrolysis. Via molecular modeling it was attempted to design phosphoramidate structures that would be more suitable for oral administration, in terms of water solubility, partition coefficient and stability in blood (Rumley-Van Gurp, 2000). Several candidate compounds were identified from this study, were synthesized and tested in vitro. Some of these appeared to be poor inhibitors of AChE. The most promising compound, 2,5-dichlorophenyl 2,2,2-trifluoroethyl phosporamidate, was tested in vivo in guinea pigs, but did not offer any protection against soman (Langenberg *et al.*, in preparation). These results, as well as the fact that phosphoramidates are not anywhere near registration as pharmaceuticals, resulted in the decision to end this line of research.

3.2 Nerve agent scavengers

The toxicity of OP compounds is due to the fact that they inhibit the enzyme AChE in an irreversible way. This leads to an accumulation of ACh and as a result to over-stimulation of the cholinergic receptors. The current prophylaxis against OP intoxication is based on the protection of part of the enzyme ChE by a reversible inhibitor. The drawback of this procedure is that this type of compounds may lead to undesired side effects in a therapeutically relevant dose.
Another approach to nerve agent prophylaxis is the use of so-called scavengers. The idea is to eliminate the nerve agent in the bloodstream before it can reach its toxicological target: AChE in the (central) nervous system. It may be expected that effective scavengers offer protection against both lethal and incapacitating effects of an acutely toxic dose. In addition, if a scavenger remains in circulation at an effective concentration during a relatively long period of time, the prophylaxis will, a fortiori, protect against a long term exposure to low doses of a nerve agent. It is further anticipated that bioscavengers do not induce adverse physiological effects, particularly when bioscavengers from human origin are applied. Two major classes of scavengers can be distinguished, i.e, stochioimetric and catalytic scavengers.

3.2.1 Stoichiometric scavengers

Nerve agents are known to bind covalently to esterases, such as AChE (E.C. 3.1.1.7), butyrylcholinesterase (BuChE, E.C. 3.1.1.8) and carboxylesterases (CaE, E.C. 3.1.1.1). This implies that these naturally occurring human enzymes can be used as scavengers. In recent years, the feasibility of using bioscavengers that can rapidly bind nerve agents has been studied, such as monoclonal antibodies (Lenz *et al.*, 1984; Brimfield *et al.*, 1985), fetal bovine serum AChE (Wolfe *et al.*, 1987; Ashani *et al.*, 1991; Maxwell *et al.*, 1992; Wolfe *et al.*, 1992) and human plasma butyrylcholinesterase (HuBuChE) (Ashani *et al.*, 1993; Raveh *et al.*, 1997; Allon *et al.*, 1998). Of the scavengers evaluated so far, HuBuChE has the most advantages as a potential candidate for human use. It binds rapidly with all highly toxic OP's, thus offering a broad range of protection for nerve agents. In addition, it has a long residence time in the circulation and, since it is an enzyme from human source, it should not produce any adverse immunological responses upon repeated administration into humans. Also, HuBuChE can be isolated from (outdated) human plasma or serum in large quantities. Very promising results were obtained with HuBuChE as a scavenger. The enzyme is rapidly distributed in laboratory animals, such as mice, rats, guinea pigs, and rhesus monkeys, after i.v. administration, followed by a slow elimination (Ashani *et al.*, 1993).
In addition, the enzyme is sufficiently absorbed following an i.m. administration to provide therapeutically significant blood levels over 10 – 70 h in laboratory animals, which is a prerequisite for practical application. The peak level in blood after i.m. administration amounted to 50-60% of the concentration obtained immediately after i.v. administration of the same amount of enzyme (Ashani *et al.*, 1993). HuBuChE circulates only in blood. Elevated levels of HuBuChE in extravascular compartments of the brain were not observed after i.m. injection of HuBuChE.
Prophylaxis with the enzyme resulted not only in an increase in survival of mice, rats and rhesus monkeys intoxicated (i.v.) with C(±)P(±)-soman or other nerve agents, but also in a significant alleviation of post-exposure incapacitation. An effective protection of guinea pigs against respiratory exposure to soman has recently been reported (Allon *et al.*, 1998). Moreover, since the efficacy of HuBuChE as a scavenger is based on the

inhibitory properties of the challenging agent, it can be expected that such scavengers will be effective against nerve agents having a wide variety of chemical structures.
The final aim is the use of this enzyme as a scavenger for protection of humans against acute intoxication by nerve agents. Although the results obtained in laboratory animals indicate the possible usefulness of HuBuChE as a scavenger, the information reported so far is insufficient for a thorough and quantitative description of the protective mechanism. Consequently, a reliable extrapolation of the working mechanism to human beings is not yet possible, although this will be needed for further development of the enzyme as a prophylaxis drug for application in humans.
Currently, the influence of HuBuChE on the toxicokinetics of nerve agents in guinea pigs and marmosets is being studied at TNO-PML within the context of co-operative agreement from USAMRMC (contract no. DAMD17-00-2-0032), as a part of the registration process of HuBuChE as a prophylaxis drug. The concept of using a human protein as a pretreatment bioscavenger has been expanded by using two different sources of the HuBuChE, one derived from outdated human blood and one derived from a transgenic animal source via recombinant technology. The latter approach has the potential to allow for large scale production of material at reasonable costs.
Currently enzymes from both sources are being studied for pharmacokinetics, efficacy against nerve agent intoxication and safety. Successful results in these experiments will provide the type of efficacy data in two species as required under the USFDA animal rule for advancement of the plasma derived HuBuChE and should allow for a subsequent investigational new drug submission.
A drawback of the use of stochioimetric scavengers is the fact that they are 'consumed' during their protective action, which requires administration of relatively high doses in terms of weight of such scavengers in order to obtain adequate protection. It has been estimated that approximately 130-200 mg.70 kg HuBuChE is needed to protect against a dose of nerve agent that corresponds with $1LD_{50}$ (Ashani and Pistinner, 2004), Estimation of the upper limit of human butyrylcholinesterase dose required for protection against Organophosphates toxicity: a mathematically based toxicokinetic model, Toxicological Sciences 77, 358-367. To improve the detoxification of OP agents by scavengers, several approaches have been recently used to increase the stoichiometry and in vivo effectiveness of ChEs as OP scavengers (Maxwell et al., 1999). The protection by scavengers may be improved by the administration, either prophylactically or immediately post-exposure, of an oxime. Oxime treatment would allow the reactivation of the scavenger in the bloodstream, so that more OP molecules can be detoxified. Obviously, this applies only to nerve agents that do not rapidly form aged inhibited HuBuChE. The efficacy of such a pseudo-catalytic scavenger depends on the concentration of the oxime near the active site of the enzyme, and will deteriorate with time as this concentration drops. As an example, this approach improved the *in vivo* protection of AChE that was administered to mice. The oxime HI-6 increased the stoichiometric neutralization of sarin from 1:1 to 57:1 (Caranto *et al.*, 1994).
Alternatively, protein engineering of HuBuChE resulted in a mutant enzyme with the ability to release bound nerve agent and thereby reactivate the enzyme. A double mutant was shown to reactivate soman before ageing could occur (Millard *et al.*, 1998). Furthermore, a mutant of mouse AChE was generated that was less susceptible to aging after soman inhibition which resulted in a two-fold increase in oxime-assisted detoxification of soman (Saxena *et al.*, 1997).

3.2.2 *Catalytic scavengers*

The second category of OP eliminating enzymes consists of those that catalytically hydrolyze (in some cases stereoselectively) OP's into non-toxic alkyl methyl phosphonic acids. These enzymes are so-called phosphotriesterases (PTE's). A comprehensive overview of currently known PTE's and their role in the detoxification of OP's was published by Vilanova and Sogorb (1999). More recently, Sogorb *et al.* (2004) reviewed the applications for PTE's in the prophylaxis and treatment of OP poisonings. PTE's that are currently known have been divided in two enzyme classes: organophosphorous hydrolases (OPH, E.C. 3.1.8.1) and diisopropylfluorophosphatases (DFP-ase, E.C. 3.1.8.2) which are also known as organophosporus acid anhydride hydrolases (OPAH). OPH's catalyze the hydrolysis of a wide range of OP's, but favour substrates where a P-O bond is cleaved. In contrast, DFP-ases favour substrates where a P-F or P-CN bond is cleaved. PTE's have been isolated from animal tissues and microorganisms. Although the physiological role of these enzymes is not known, a clear correlation exists between the levels of PTE's and susceptibility of the species to the toxic effects of OP's.
In addition, catalytic antibodies have been raised against appropriate nerve agent/active site transition state analogs (Brimfield *et al.* 1993, Zhao *et al.* 1997, Vayron *et al.* 2000a, b).

As early as 1957, Cohen and Warringa achieved some protection in rats against a lethal subcutaneous dose of diisopropyl phosphorofluoridate and sarin by prophylaxis with the enzyme 'fluorosphatase', nowadays known as OPH, capable of hydrolyzing organophosphates.
For therapy and/or profylaxis, a hydrolytic enzyme could be administered in smaller quantities than scavengers and could produce the same or greater degree of protection as scavengers. It would also have the advantage of not being consumed in the process of detoxifying the nerve agent, so it would be available to protect against multiple exposures of either high or low dose. Various studies have been carried out to investigate the potential of PTE's to be used for therapy and/or prophylaxis. These studies are reviewed by Sogorb *et al.* (2004). OPH from *Pseudomonas diminuta* has been shown to afford protection against soman lethality in mice and to protect against behavioral side effects (Broomfield, 1993). It was also shown to offer a higher protection against sarin in mice than pyridostigmine and physostigmine (Tuovinen *et al.*, 1999).
The OPH human paraoxonase (HuPON) has been identified as having a similar potential for affording protection against OP's, but without the complication of inducing an immune response in humans (Gan *et al.* 1991, Broomfield 1993, Masson *et al.* 1998, Mackness *et al.* 1998). HuPON hydrolyzes OP insecticides and nerve gases and a relationship was found between the amount of enzyme in different species and the toxic response towards OPs. The enzyme has not yet been tested for efficacy in OP protection in a mammalian model system. Although PTE's possess the desired catalytic activity, the activity is considered insufficient for use as a nerve agent pretreatment. Therefore, researchers are attempting to improve the activity through protein engineering by specific mutation of HuPON (Masson *et al.*, 1998).
The injection of exogenous proteins directly into the blood could result in immunological problems. Moreover, exogenous PTE's are quickly inactivated through protease action. Several approaches have been developed to overcome these problems (Sogorb *et al.* 2004).
Possibly, surface modifications in recombinant bacterial enzymes could reduce immunogenicity. Another approach is to use a carrier that isolates the PTE's from the

internal media. This carrier must supply a 'bioprotective' environment and be permeable to OP's. The carriers studied so far are erythrocytes and stabilized liposomes.
As an alternative for the injection of enzymes, the possibility of extra-corporeal therapy is being considered (Masson *et al.*, 1998). Thus, blood could be detoxified by circulation through a reactor containing immobilized PTE.

The search for (mainly) bacterial OPH's is being continued. A problem associated with OPH's is that they predominantly hydrolyze the relatively non-toxic stereoisomers of nerve agents (Benschop and De Jong 1991), which does not contribute substantially to the detoxification process. Furthermore, such enzymes need to be 'humanized' before they can be used in man, upon which most often the catalytic activity is reduced by several orders of magnitude (Lenz, personal communication 2002).
As discussed above, PTE's have been identified with catalytic activity towards various OP's that contain P-O, P-F, or P-CN bonds. However, it was observed that OP's containing a P-S bond, such as VX, are more resistant to enzymatic hydrolysis. The only yet identified PTE that detoxifies VX was isolated from *Pseudomonas diminuta* (Rastogi *et al.*, 1997, Kolakowski *et al.*, 1997). The enzyme was shown to hydrolyze a variety of OP's, containing P-O, P-F, P-CN and P-S bonds. However, the observed hydrolysis of VX was much slower than that of other OP's. Attempts are currently being made to modify the cloned gene for this enzyme, to change the catalytic specificity and to increase the ability to degrade OP's (diSioudi *et al.*, 1999; Gopal *et al.*, 2000).

During the study of the toxicokinetics and metabolism of (±)-VX within the context of USAMRMC Co-operative agreement DAMD17-97-2-7001 Van der Schans (2000) observed an apparent catalytic hydrolysis of the most toxic VX-isomer in homogenate of human and marmoset liver, in contrast to that of the guinea pig. A follow-up of this intriguing finding has taken place within the context of R&D Program V013. Preliminary conclusion of that study is that this is probably a pseudo-catalytic hydrolysis: covalent binding of VX to a CaE isoenzyme followed by spontaneous reactivation (Boone and Van der Schans 2004). These findings are supported by a paper of Maxwell and Brecht (2001), who demonstrated that rat plasma CaE shows spontaneous reactivation after inhibition with OP's, with the highest reactivation rate after inhibition with VX. The enzyme in primate liver is the first that has a preference for hydrolysis of the most toxic enantiomer of (±)-VX. Purification of the enzyme is necessary for a thorough study of the properties of the enzyme and to investigate the potential to use the enzyme for nerve agent prophylaxis or other purposes.

3.3 Physostigmine as an alternative for pyridostigmine

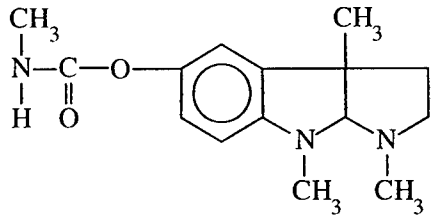


Figure 4 Structural formula of physostigmine.

Physostigmine (Figure 4) is derived from the seeds of the calabar bean Physostigma venenosum growing in West Africa. Daniell, a British medical officer, brought this bean to Engeland in 1840. It is an alkaloid that inhibits AChE. This inhibition was first established in 1946. It was shown that after prophylaxis of cats with a small dose of eserine the animals could withstand several times the LD_{50} of diisopropyl-phosphorofluoridate (DFP) (Koster, 1946). Physostigmine has also been used clinically. The first therapeutic use of physostigmine was in the treatment of glaucoma by Laqueur in 1877. Another therapeutic application was the improvement of memory functions in patients with Alzheimer's disease (Muramoto *et al.*, 1984; Whitehouse, 1993; Thal *et al.*, 1983). Furthermore, physostigmine can reverse the toxic effects associated with anticholinergic poisoning (Rumack, 1973) or with an overdosage of other drugs (Nattel, 1979). In the latter study physostigmine restored vital functions until the drugs were eliminated from the body. Physostigmine is used to compensate overdosage of tricyclic antidepressants (Di Liberti *et al.*, 1975; Larson *et al.*, 1977), morphine (Weinstock *et al.*, 1981) and benzodiazepines (Bernards, 1973). Because the carbamate physostigmine, like pyridostigmine, reversibly inhibits AChE, it could replace pyridostigmine as a prophylactic treatment. For being an effective prophylaxis, physostigmine should meet the earlier mentioned three conditions.

3.3.1 Protection with physostigmine against lethality

It has been shown that physostigmine pretreatment is effective against OP poisoning: a significant protection against lethality after sarin and soman intoxication has been reported (Leadbeater *et al.*, 1985). This compound has also proved to be more effective than pyridostigmine in preventing toxic effects after soman poisoning in guinea pigs and rats (Solana *et al.*, 1990; Miller *et al.*,1993).

After an acute physostigmine prophylaxis a protective ratio against lethality in guinea pigs of 2.5 after soman poisoning and of 14.4 followed by a post intoxication therapy was reported. In another study subchronic physostigmine (0.0048 mg/hr) prophylaxis for 6 days prior to LD_{99} soman offered a survival rate of 4/8 and when combined with scopolamine (0.0019 mg/hr) of 8/8 (Wetherell, 1994). Lallement *et al.* (2001) also found a better protection against early mortality after soman intoxication with the combination of physostigmine and scopolamine pre-treatment compared with pyridostigmine or physostigmine alone.

At TNO the efficacy of different prophylaxis protocols with physostigmine in protecting animals against lethality after soman poisoning were investigated. To examine the protective efficacy of the prophylaxis against OP induced lethality physostigmine was administered prior to soman intoxication. The protection of four different prophylaxis regimes followed by a post-intoxication therapy of atropine against $3xLD_{50}$ soman intoxication was tested and compared in the guinea pig

(Philippens *et al.*, 1998). The prophylaxis scenarios consisted of: a single dose of physostigmine or pyridostigmine combined with scopolamine given 30 minutes prior to soman, or subchronic physostigmine alone or in combination with scopolamine for 10 days prior to soman. In this study an atropine sulphate injection was given one minute after soman. In another study with guinea pigs, the addition of scopolamine to the prophylaxis was tested with or without a post-intoxication therapy of atropine sulphate (Philippens *et al.*, 2000b). Pretreatment with physostigmine and scopolamine was also tested in the guinea pig in a more realistic situation. A chronic stress model was added in the experimentation set-up. In this study the animals were intoxicated with 2x LD_{50} soman followed by atropine sulphate. Furthermore, the protection of subchronic physostigmine prophylaxis in combination with a post-intoxication therapy of atropine against 2x LD_{50} soman intoxication was tested in the marmoset monkey and compared with the efficacy of the post intoxication therapy of atropine in nonpretreated animals (Philippens *et al.*, 2000a).

A high protective efficacy against soman induced intoxication is reported in both guinea pigs and marmoset monkeys. Guinea pigs that were pretreated with a single dose of physostigmine in combination with scopolamine and atropinised one minute after intoxication with 3xLD_{50} soman all survived (Philippens *et al.*, 1998), whereas only 43% of the guinea pigs pretreated with a comparable dose of pyridostigmine (0.04 mg/kg sc) in combination with scopolamine survived (Philippens *et al.*, 1998). These findings suggest that physostigmine is a more effective prophylaxis than pyridostigmine. This is in accordance with earlier reported findings (Solana *et al.*, 1990; Miller *et al.*, 1993). These investigators compared the efficacy of pyridostigmine and physostigmine prophylaxis against soman intoxication in guinea pigs and rats respectively and found a better protective effect against lethality after physostigmine prophylaxis. The protective efficacy of pyridostigmine reported in our studies (Philippens *et al.*, 1998; Philippens, 2002 not published) was worse compared with the one reported by Gordon *et al.*(1978). They found a protective ratio of 8.0 with a high dose of pyridostigmine (0.10 mg/kg im) against soman intoxication. Since these investigators applied pyridostigmine im instead of sc different pharmacokinetics may explain the difference in protective efficacy. Such a difference was also found for the phosphoramidate PNF. When this cholinesterase (ChE) inhibitor was administered iv 30 minutes prior to soman intoxication a high protective ratio was found (Langenberg *et al.*, 1996), whereas a sc injection with the same pre-dose time leading to a comparable AChE-inhibition did not protect against soman induced lethality (Melchers *et al.*, 1994). This suggests that for effective protection against lethality the route of administration is important, most likely because these different routes of administration may lead to different carbamate or phosphoramidate plasma levels. A chronic transdermal application of physostigmine offering a ChE inhibition of 53% leads to a plasma level of 4.1 $\pm$ 0.8 ng/ml, while a single im injection that offers almost the same ChE inhibition of 59% leads to a plasma level of 14 $\pm$ 1.3 ng/ml (Meshulam *et al.*, 1995). This is due to the kinetic. Shortly after a single i.v. administration the plasma level is at its maximum (no absorption), whereas after a s.c. administration the maximum plasma level will appear at the time that the absorption is equal to the excretion. Some of the compound will bind to the enzyme, some of the compound will bind to other targets (like receptors) and some of the compound is still free. When the compound is injected immediately in the blood stream the unbound compound will be highest. Therefore, single iv injection will cause a high plasma level of the compound immediately after injection. Such an accumulation of free carbamate in the circulation will direct the carbamoylation reaction (binding of the enzyme with the carbamate) to the right leading

to a high rate coefficient for decarbamoylation of carbamoylated enzyme (reactivation of the enzyme)(Watts and Wilkinson, 1977). When a slow release strategy is followed the reaction will reach a steady state: the rate coefficient for carbamoylation of the enzyme from the enzyme/carbamate complex will be almost equal to that for decarbamoylation of carbamoylated enzyme, preventing early reactivation of the protected enzym. This situation is aimed at the current pre-treatment that consists of repeated oral pyridostigmine administration. From the fore mentioned it may be clear that the protection against lethality obtained by a single injection of pyridostigmine or physostigmine can not predict the efficacy that will be obtained after continuous administration. For that reason the efficacy against lethality of a more chronic application should be considered and experimentally evaluated. Therefore, physostigmine subchronically administered by an osmotic mini pump at a therapeutically relevant dose, alone or in combination with scopolamine, was tested for its efficacy in guinea pigs (Philippens *et al.*, 1998). These animals were, like those that were pretreated with a single dose of physostigmine at a time point analogous with the inhibition of enzyme during the chronic administration, protected against a 3x LD_{50} soman intoxication. This finding corroborates an earlier report that described a similar protection of acute and subchronic physostigmine, both combined with scopolamine, against the toxic influences of 2 or 5x LD_{50} soman in guinea pigs (Anderson *et al.*, 1991). Furthermore, chronic daily stress factors for 8 weeks did not affect the protection against lethality in guinea pigs (Philippens *et al.*, 2005). To simulate the influences of a war situation, the stress factors were chosen to be unpredictable, uncontrollable and variable. Therefore, physostigmine seems to fulfill the first condition of a successful prophylaxis. To increase the probability that extrapolation of these data to man is allowed, this procedure was also examined in the marmoset monkey (Philippens *et al.*, 2000a). After subchronically administered physostigmine, even without the addition of scopolamine, marmoset monkeys were completely protected against lethality induced by 2x LD_{50} soman (sc) intoxication, provided a therapy with atropine sulphate was given. A comparable effect was found in a study with cynomolgus monkeys *(Macaca fasicularis)* (von Bredow *et al.*, 1991). In this study physostigmine with scopolamine was also effective against an intoxication with 5xLD_{50} soman followed by atropine and 2-PAM therapy.

Summarized, physostigmine pretreatment protects better than pyridostigmine pretreatment against soman or sarin intoxication measured in rats and guinea pigs. Physostigmine with a post-intoxication therapy of atropine sulphate was also effective in marmoset monkeys. In case no post-intoxication therapy with atropine sulphate was given, the addition of scopolamine improved the efficacy against lethality. The efficacy of the combination of physostigmine and scopolamine was also better than the combination of pyridostigmine and scopolamine in guinea pigs.

3.3.2 *Side effects of physostigmine*

In contrast to pyridostigmine the molecular structure of physostigmine contains a tertiary nitrogen atom, that allows this molecule to pass the blood-brain barrier (see Figures 3 and 4) and exert effects at both peripheral and central cholinoceptive sites. When taken for a prolonged period unwanted side effects may be expected to develop. Most side effects of physostigmine pretreatment can be expected to be centrally mediated effects. Depending on the affected brain areas, these effects can induce changes in different types of behaviour. In studies of Alzheimer's disease, a lot of research has been carried out with respect to physostigmine. Focusing on the clinical symptoms, physostigmine leads to dose dependent central and peripheral effects like

hypersalivation, hypothermia, miosis and tremors (Yoshida and Suzuki, 1993). Subchronic physostigmine (0.12 mg/kg/hr) in the guinea pig, leading to blood AChE inhibition of 20-40% and brain AChE inhibition of 20%, did not affect the body temperature and water consumption and induced no tremors (Lim *et al.*, 1988a). Also effects in behavioural test systems were described after physostigmine: in an operant conditioning chamber a decrease in performance was found after physostigmine (0.4 mg/kg sc in the rat) (Genovese *et al.*, 1990). The antimuscarinic scopolamine was able to antagonise this effect completely. In cases of subchronic physostigmine treatment, leading to blood AChE inhibition of 42%, no effects on the motor performance measured by an accelerating rota-rod was found (Harris *et al.*, 1989). On neurophysiological level, physostigmine (1 mg/kg) modulated the visual evoked potential (VEP) of the rat in a behavior-related manner: all the VEP peaks changed conformable with a high arousal behavioral state (Bringmann, 1994).

It may be clear that drugs when given prophylactically to healthy persons should be devoid of side effects, in particular, when these drugs have to be taken for several weeks or even months. Since physostigmine also inhibits AChE in the brain, the accumulation of ACh could lead to unwanted central side effects. Most of these side effects can be counteracted by a cholinolytic drug. In most of the published studies a cholinolyticum was added to the prophylaxis to acquire a better protection against OP intoxication (Leadbeater *et al.*, 1985; Lim *et al.*, 1988b; Meshulam *et al.*, 1995). A cholinolyticum is also one of the drugs applied as post intoxication therapy to prevent further over-stimulation of the cholinergic receptors. In our studies the centrally active cholinolyticum scopolamine is added to the prophylaxis regime to prevent physostigmine-induced side effects (Philippens *et al.*, 1992; Philippens *et al.*, 1996; Philippens *et al.*, 2000b). The high penetration into the brain makes this compound well suited to counteract the side effects of physostigmine. Indeed, scopolamine is better in antagonizing the physostigmine-induced behavioral suppression than atropine (Genovese *et al.*, 1990). Fortunately, the prophylactic use of scopolamine does not interfere with the post-intoxication therapy with atropine (Philippens *et al.*, 2000b). The antagonising capacity of scopolamine is also described in our study (Philippens *et al.*, 1996): scopolamine was shown to prevent unwanted behavioural and neurophysiological side effects. Not all the side effects caused by a single dose of physostigmine could be prevented by scopolamine. It could be demonstrated that some effects were due to a direct action of physostigmine on nicotinergic receptors and not related to the inhibition of AChE (Philippens *et al.*, 1997). This is discussed in paragraph 3.4.

Fortunately, no side effects were observed during subchronic physostigmine and scopolamine pretreatment in marmoset monkeys on task accuracy and response rates in a cognition task (Muggleton *et al.*, 2003). Even without scopolamine, subchronic physostigmine pretreatment did not lead to any side effects on hand-eye coordination, startle reflex and loco-motor activity in the marmoset monkey (Philippens *et al.*, 2000). Comparable results were found in the guinea pig (Philippens *et al.*, 1998). One possible explanation could be the development of tolerance against AChE inhibitors. However, the absence of side effects was also observed in the startle reflex: these results were considered not to be related to AChE inhibition (Philippens *et al.*, 1997). Presumably, adaptation also occurred on nicotinergic receptors sites, since soman may act on nicotinergic and muscarinergic receptors, thereby amplifying the protecting efficacy of physostigmine. The finding that a more practical administration of physostigmine by subchronic application did not lead to side effects suggests the use of a cholinolyticum

to be redundant. This is corroborated by the results observed in the study where marmoset monkeys were used instead of guinea pigs (Philippens *et al.*, 2000 a). In the marmoset primate the subchronic administration of physostigmine alone, at a dose leading to an AChE inhibition of 30%, did not lead to physical, behavioural and neurophysiological side effects. This is in accordance with a study performed with rhesus monkeys: physostigmine at a dose of 50 ug/kg (im), with a blood ChE inhibition of approximately 30%, did not lead to any side effects on physiological parameters like heart and respiratory rate and brain activity (Jeevaratnam *et al.*, 1998). These studies were performed in standard laboratory circumstances. In a war situation stress factors may be expected. In case animals were exposed to chronic stress during the prophylaxis period with physostigmine also no unwanted and serious side effects in guinea pigs were found (Philippens, unpublished results 2000). During the first three weeks stress alone had a positive effect on the activity and exploration. During the prophylaxis period also an increase of exploration activity was found.

Summarized, concerning the prevention of unwanted side effects one can conclude that a subchronic application of physostigmine offers the best results, also under stress circumstances. Most expecting effects will be due to AChE inhibition. Some effects may be due to direct effects on e.g. nicotinic receptors by physostigmine. To prevent side effects due to AChE inhibition of the prophylaxis, the addition of scopolamine as a supplement to the pretreatment regime may be necessary in case a bolus injection of the pre-treatment is given instead of a chronic steady state application.

3.3.3 Post-intoxication incapacitation in physostigmine pretreated animals

A successful prophylaxis should not only protect against lethality but also against the incapacitation due to the OP intoxication. Most of these incapacitating effects are caused by the high AChE inhibition and are manifested by muscarinic and nicotinic peripheral and central signs. As mentioned before, physostigmine easily penetrates the brain and, therefore, should protect the CNS against the toxic influences of OPs. Following soman intoxication (LD99) guinea pigs exhibited signs of OP poisoning like hyperactivity, chewing, tremors, prostration, and salivation. Guinea pigs pretreated with subchronic physostigmine (offering a blood AChE inhibition of 26%) showed no obvious signs of poisoning after this dose of soman at 4 h post-intoxication, whereas in the guinea pigs pretreated with the combination of subchronic physostigmine and scopolamine the signs already had disappeared 2 h post-intoxication (Wetherell, 1994). The protective ratio in guinea pigs against post soman intoxication incapacitation, measured by gross motor performance in a swimming test, was found to be 2.0 after physostigmine pretreatment and 1.0 after pyridostigmine pretreatment (both in a comparable dose leading to a high peak erythrocyte AChE inhibition of 70%) (Leadbeater *et al.*, 1985). This is in accordance with another study in rats: subchronic physostigmine prophylaxis protected significantly better against soman induced incapacitation than subchronic pyridostigmine prophylaxis (Miller *et al.*,1993).

Post-intoxication incapacitation can be measured by observing the first characteristic signs and symptoms of intoxication (Wetherell, 1994; Miller *et al.*, 1993; Solana *et al.*,1990). These symptoms can offer a good indication of how effective a prophylaxis is in reducing post intoxication incapacitation. Through comparison of different prophylaxis regimes it was already shown in rats that a subchronic pretreatment with physostigmine protected significantly better against soman induced intoxication symptoms than pyridostigmine (Miller *et al.*, 1993). But, when these symptoms have disappeared the animals may still suffer from the OP intoxication. These incapacitating

effects may influence the soldier's performance and therefore need further evaluation. At TNO Defence, Security and Safety animal studies are reported that measure post intoxication incapacitation objectively by using behavioral and neurophysiological read-out systems. Below the symptoms (clinical signs) and behavioural effects are described.

3.3.3.1 Clinical signs

In one of our studies (Philippens *et al.*, 1998) different applications and combinations of physostigmine prophylaxis were tested and compared in counteracting soman-induced symptoms in atropinized guinea pigs. The order of protective efficacy was: acute physostigmine+scopolamine > subchronic physostigmine > subchronic physostigmine+scopolamine. The better protective effect of the acute prophylaxis can be explained by the higher AChE inhibition, since a relation has been reported between AChE inhibition and protecting efficacy against OP intoxication (Harris *et al.*, 1989). In case of the sign free subchronic prophylaxis regimes, the treatment with physostigmine alone seemed to offer the best protection against 3x LD_{50} soman induced symptoms (Philippens *et al.*, 1998). A clear protection of subchronic physostigmine was also observed after 2x LD_{50} soman in marmoset monkeys (Philippens *et al.*, 2000 a). However, guinea pigs that had received a pretreatment with physostigmine and scopolamine appeared to recover faster (Philippens *et al.*, 2000 b). This was in accordance with other investigators: Guinea pigs pretreated with subchronic physostigmine showed no obvious signs of poisoning at 4 h post-intoxication, whereas in the guinea pigs pretreated with the combination of subchronic physostigmine and scopolamine the signs already had disappeared 2 h post-intoxication (Wetherell, 1994). All these studies were performed under standard laboratorium conditions. In a more realistic war situation soldier will be exposed to stress factors. Stress may influence the side effects and efficacy of the pretreatment. If guinea pigs were chronically exposed to unpredictable, uncontrollable and variable stress factors before and during the prophylaxis with physostigmine and intoxication with soman the typical signs of intoxication, including convulsions responsible for brain damage, were worse than animals treated under standard conditions (Philippens *et al.*, 2005). However, these circumstances did not affect the lethality.

3.3.3.2 Behavioural and neurophysiological effects

When the first signs of intoxication have disappeared the animals may still suffer from the OP intoxication. This is most accurately reflected by behavioural and neurophysiological parameters. Because physostigmine easily penetrates the brain it should protect the CNS against toxic influences of OPs. Indeed, the incapacitation protective ratio in guinea pigs against post soman intoxication incapacitation, measured by gross motor performance in a swimming test, was found to be 2.0 after physostigmine pretreatment and not found after pyridostigmine pretreatment (PR on the swimming task was 1.0) (both at a comparable dose leading to a high peak erythrocyte AChE inhibition of 70%) (Leadbeater *et al.*, 1985). The behavioural performance in different test systems and the spontaneous and evoked brain activity were measured after the most severe symptoms of soman (2x LD_{50}) intoxication had disappeared to study post-intoxication incapacitation (Philippens *et al.*, 2000b). The shuttle box performance was not affected in the guinea pigs that were pretreated with subchronic physostigmine and scopolamine, whereas, the guinea pigs that received only subchronic physostigmine prophylaxis showed a decline in their performance and were not able to recover in this task within a week (both groups received an atropine sulphate injection one minute after soman). On the other hand this latter group responded normal again in

the startle response task 24 hours after soman, while in the physostigmine/scopolamine/atropine and the physostigmine/scopolamine groups it took 24 hours longer. Albeit, the effects on the startle reflex in the physostigmine/scopolamine were not found to be significant. Similar post soman intoxication effects were observed in subchronic physostigmine pretreated marmoset monkeys in the startle reflex (Philippens *et al.*, 2000a). These animals performed almost normally in all the tasks shortly after soman intoxication. Also high cognitive function (discrimination reversal task) was restored with physostigmine and scopolamine in the marmoset monkey after $0.5xLD_{50}$ sarin or soman (Muggleton *et al.*, 2003). Physostigmine and scopolamine was also tested against $2.5xLD_{50}$ sarin in beagle dogs. Twenty minutes after intoxication these animals were fully recovered (Meshulam *et al.*, 2001). However, no control values are present to compare this pretreatment with pyridostigmine in this species. In two studies with rhesus monkeys the effects after soman on behavior was tested in physostigmine pretreated animals (Blick *et al.*, 1993) or in pyridostigmine pretreated animals (Blick *et al.*, 1991) (both leading to a serum cholinesterase inhibition of 30-60%). Physostigmine seemed to be slightly protective against repeated low-dose exposures of soman (5-day soman ED50), while pyridostigmine, on the other hand, worsened the effects of the post-intoxication signs after soman. The soman ED50 in the control animals was lower than in pretreated animals. Blick et al. (1994) reconfirmed the findings that pyridostigmine did not prevent soman-induced performance decrements in rhesus monkeys, even though pyridostigmine is effective in preventing death after much higher doses.

Summarized, to prevent or reduce the post intoxication incapacitation, one can conclude that the subchronic prophylaxis with physostigmine or physostigmine and scopolamine offer both a high reduction of the incapacitation after $2x\ LD_{50}$ soman in case a post intoxication therapy with atropine is used. Pretreatment with pyridostigmine was not able to prevent post-intoxication incapacitation.

3.4 Compounds with another mechanism of action than cholinesterase inhibition

It is assumed that the protection of carbamates like pyridostigmine and physostigmine is based on the reversible inhibition of the enzyme AChE. However, of physostigmine it is known that it can affect receptors in a direct manner (Albuquerque *et al.*, 1984; Albuquerque *et al.*, 1988; Sherby *et al.*, 1984, Van den Beukel *et al.*, 1998a, Van den Beukel *et al.*, 1998b). The ED_{50} of physostigmines' agonism at the nicotinergic receptor even appears to be lower than its IC_{50} of AChE inhibition (Albuquerque *et al.*, 1988). Interestingly, nerve agents and in particular VX and soman also affect ACh receptors (Bakry *et al.*, 1988). Bakry et al. reported that soman at micromolar concentrations can act as a partial agonist of the nicotinergic ACh receptor and can induce receptor desensitisation. At lower concentrations VX and soman may also affect a small population of muscarinergic receptors that show the same affinity for the OP compound as AChE. This observation suggested that the toxicity of soman might involve a combined action on nicotinergic and muscarinergic receptors. The direct effect of physostigmine on nicotinergic receptors can therefore be of importance for the protecting efficacy of physostigmine. Very interesting results were found in a study in which the optical isomer of physostigmine, (+)-physostigmine that not or hardly inhibit ChE, was compared with the regularly used (-)-physostigmine. (+)-Physostigmine did not inhibit and therefore did not protect AChE, whereas the symptomatology, like chewing, salivation, ataxia, muscle fasciculations, tremors, convulsions, and dyspnoea, and the post-intoxication incapacitation after soman ($2xLD_{50}$) were comparable between both pretreatment protocols (Philippens, unpublished results 2001). Therefore, studying the mechanisms of the effects of physostigmine will learn us more about its protective effect.

Furthermore, besides the cholinergic system, other transmitter systems may play a role in the protection against OP intoxication. Communication in the central nervous system is more complex than information flow along single transmitter systems. Nerve cell terminals can possess different receptor types, which can be from different transmitter systems. Therefore, cells with different transmitter systems can be connected with each other. Furthermore, drugs can have more than one mechanism of action. It is known that adenosine plays a functional role in nervous tissue as a regulator of neural activity: it inhibits neural firing and release of acetylcholine (Spignoli et al., 1984). The typical sign of yawning after ChE inhibition can be counteracted by adenosine receptor agonists (Zarrindast et al., 1995). Furthermore, most adenosine receptors are located in the striatum (Bruns et al., 1986), which also regulate ACh release. The GABAergic system, which is the main inhibitory system in the brain, also regulates neural activity. Centers that are under control of the GABAergic system are the amygdale and hippocampus (Izquierdo and Medina, 1991). These centers are rich in cholinergic synapses and take part in memory formation. Indeed, GABAergic antagonists and agonists influence the physostigmine induced learning effects, while these drugs by themselves had no effect on the learning task (Zarrindast et al., 1998). It seems that the NMDA receptor complex also plays an important role in the protective effect of anticholinergic drugs against soman poisoning (Raveh et al., 1999). This effect is based on the fact that uncontrolled and progressive seizure activity after soman intoxication, recruited other neurotransmitter systems like the NMDA system. Indeed, procyclidine, a drug with anticholinergic and anti-NMDA activity, in combination with physostigmine and a post-intoxication therapy with atropine sulphate and HI-6 offer a very high protection ratio of 21.5 in guinea pigs against GD. Without the post-intoxication

therapy the protection ratio was still 4.7 (Choi *et al.*, 2004). This was confirmed by a study of Myhrer *et al.* (2004a) in which the dose related protection of physostigmine and procyclidine was studied in rats. These therapeutic doses of physostigmine and procyclidine did not lead to cognitive side effects in rats (Myhrer *et al.*, 2004b)
In a not yet published pilot study (Philippens, 2002) at TNO with guinea pigs we found a 100% protection after physostigmine and procyclidine against soman (2x LD_{50}). In the same study the combination of physostigmine with scopolamine led to a protection of 75% survivals, whereas, the animals pretreated with pyridostigmine all died. Therefore, procyclidine is an interesting compound for further evaluation as an alternative for the current prophylaxis with pyridostigmine against OP-intoxication.

4 The ideal regime for prophylaxis with respect to OP intoxication

Concerning the prerequisites of a successful prophylaxis the subchronic prophylaxis with physostigmine alone seems to be a better alternative for the current prophylaxis with pyridostigmine. Some important results of side effects, efficacy against lethality and postintoxication incapacitation collected at TNO Defence, Security and Safety with physostigmine and scopolamine are shown in Table 3.

Because physostigmine can easily penetrate into the brain, the risk of side effects will be high. To suppress these undesired side effects, a low dose of the muscarinic antagonist scopolamine can be added to the prophylaxis (Koplovitz *et al.*, 1995; Leadbeater *et al.*, 1985, Philippens *et al.*, 1992; Philippens *et al.*, 1996). Scopolamine in a dose of 0.1, 0.2 or 0.4 mg/kg had no effect on the startle reflex, and the dose 0.1 mg/kg had also no effect on a memory task (Philippens et al., 1996). The protocol of transdermal co-administration of physostigmine and scopolamine was also tested on side effects in human volunteers by colleagues of the UK. This application led to an increase of 13.3% at the first day to 37.7% blood-AChE inhibition on the seventh day. Beside the skin irritation on the lower chest wall due to repeated re-application of patches to the same site, which was probably also the cause of increase in drug delivery, no significant side effects were observed during a seven days experiment with 16 volunteers (placebo: n = 8; pre-treatment: n = 8). The side effects were tested by cognitive and psychomotor performance, self evaluation, physiology and vision. However, effects on vision were reported after removal of the last patch. This can be the result of adaptation processes.

The co-administration of physostigmine and scopolamine results in combination pharmacology. Is additional therapy with scopolamine necessary to antagonize side effects and improve the efficacy of the prophylaxis? In the previous paragraphs, animals pretreated with physostigmine alone combined with a post-intoxication therapy with atropine sulphate offer a good protection against soman intoxication. However, it was reported that a prophylaxis with a carbamate alone did not offer any protection against lethality (Lim *et al.*, 1988b; Gordon *et al.*, 1978). This was confirmed by a study with guinea pigs by Philippens *et al.* (2000b) in which the efficacy concerning the survival and post-intoxication incapacitation of subchronic physostigmine prophylaxis with or without the addition of scopolamine was tested in combination with a post-intoxication therapy of atropine or without an additional therapy. All animals pretreated subchronically with physostigmine alone, without any additional therapy, died; the animals that received a post intoxication therapy with atropine survived after 2x LD_{50} soman intoxication. Furthermore, animals that received the complete prophylaxis (subchronic physostigmine/scopolamine) without a post-intoxication therapy also survived. In this situation the addition of scopolamine in the prophylaxis regime is of vital importance. Interestingly, the treatment with a cholinolytic alone did not protect against soman induced lethality. Atropine alone has a protective ratio of only 1.5 against soman intoxication (Inns and Leadbeater, 1983), and scopolamine alone did not offer any protection (Wetherell, 1994).

Table 3 Overview of the data obtained with different prophylaxis regimes: appearance of side effects during prophylaxis and protection against soman intoxication followed by atropine therapy[a].

Pre-treatment	AChE inhibition	Side effect	soman xLD_{50}	Mort-ality	Intoxication symptoms		Intoxication incapacitation
					Conv.	Dyspnoea	
Guinea pig							
Acute PYR	24.8	NT	3	4/7	4/7	5/7	NT
			1.5	0/8	0/8	0/8	>5
Acute PHY	44.5	++	3	0/6	1/6	0/6	NT
			1.5	0/8	0/8	0/8	<1
Acute PHY/SCO	50.5	+	3	0/8	1/8	1/8	NT
Subchr. PHY/SCO	33.3	-	3	2/8	7/8	5/8	NT
	21.2	-	2	0/6	2/6	0/6	<1
			2[b]	1/6	4/6	5/6	>7
Subchr. PHY	35.8	-	3	0/5	2/5	1/5	NT
	20.3	-	2	0/7	3/7	0/7	7
			2[b]	6/6	5/6	6/6	NT[c]
Marmoset monkey							
Subchr. PHY	30.7	-	2	0/6	0/6	0/6	7
Control	0	-	2	3/3	3/3	3/3	NT

a) Atropine therapy was given im one minute after soman (sc) intoxication (guinea pigs: 17.4 mg/kg , marmoset monkeys 5 mg/kg).

b) No post-intoxication therapy with atropine was given.

c) All animals in this group died. Therefore, it was not possible to test the incapacitation.

The degree of side effects during prophylaxis is expressed as follows: -: not observed, +: observed in 50% of the used read-out systems, ++: observed in all read-out systems. The mortality and intoxication symptoms are expressed as the number of animals per treatment group in which these effects occur.

Conv: convulsions. The post intoxication incapacitation was expressed as the maximal number of days after intoxication that effects were detectable in behaviour.

PHY: physostigmine, PYR: pyridostigmine, SCO: scopolamine, NT: not tested. (Philippens et al., 1996; Philippens et al., 1998; Philippens et al., 2000a; Data about pyridostigmine were collected from an internal report by Philippens, 1993).

A prophylaxis regime that fully protects against lethality and post intoxication incapacitation not only induced by soman but also by many of the known nerve gases would solve the problems that still consist in the oxime therapy. This was stated by Wolthuis *et al.* in 1981 but is still valid. Therefore, the combination of physostigmine and scopolamine was tested in guinea pigs against soman, sarin, tabun, or VX. This pre-treatment protocol protected also 100% against tabun, sarin, and VX. In contrast with the soman and sarin exposed guinea pigs, the tabun and VX exposed animals did not suffer from convulsive activity. Furthermore, the animals exposed to sarin and tabun

were able to have a high performance in a memory task 3 hours after the intoxicant (not published data, Philippens, 1999). All these animals were treated after the nerve agent with a small dose of atropine sulphate (0.36 mg/kg im). In a study of Wetherell et al. (2002) the efficacy of the sub-chronic pretreatment with physostigmine and hyoscine (scopolamine) was compared to the current used pretreatment with pyridostigmine in guinea pigs. The efficacy was tested against 1.25 x LD_{50} of GB, GD, GA, GF, or VX. Physostigmine and hyoscine showed a much better protection against all nerve agents compared to pyridostigmine. In this study a significant better protection against GB, GD and VX lethality was found (Wetherell *et al.*, 2002).

For the time being, it can be concluded that subchronic prophylaxis with physostigmine and scopolamine seems to be the best choice and most promising alternative to prevent OP intoxication, although some post intoxication effects on the startle reflex still exist (Philippens *et al.*, 2000b). These effects may be related to direct effects on nicotinergic receptors. In that case, the addition of a nicotinergic antagonist would be advisable to further improve the efficacy of the prophylaxis regime. In a previous study with guinea pigs (not published) we found that the addition of mecamylamide to the prophylaxis prevent the animals from convulsions and dyspnoea which led to less post-intoxication incapacitation. In contrast to pyridostigmine, oral delivery of physostigmine is not practical due to high first pass metabolism and short elimination half life. Therefore, a more practical route of administration should be selected. On possibility is sustained release of physostigmine with an injectable polymeric microparticle system (Chaw *et al.* 2003). Another method of choice is the transdermal administration of physostigmine by using plaster pads on the skin. This approach seems to be effective in guinea pigs (Meshulam *et al.*, 1995) and leading to no side effects in human volunteers (Walter *et al.*, 1995).
For the further future, research towards compounds with another mechanism of action should be considered. Especially the anti-Parkinsons' Disease drug procyclidine. This compound is, like physostigmine, already registered as a drug for medical use. Therefore, procyclidine is a very interesting compound for further evaluation as an alternative for a prophylaxis against OP-intoxication.
And for the longer term the use of scavengers (preferably catalytic) can be evaluated.

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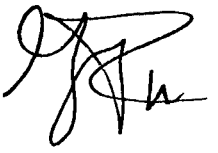

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15. ABSTRACT (MAXIMUM 200 WORDS (1044 BYTE))

Prophylaxis against chemical warfare agents may reduce the extent of the intoxication and thereby improve the prognosis for the patient. Since treatment for intoxications with organophosphorous (OP) acetylcholinesterase (AChE) inhibitors is still far from ideal, research efforts are devoted towards finding an effective prophylaxis. A successful prophylaxis should fulfil 3 conditions: 1) offering a high protection rate, 2) causing no side effects, and 3) protecting against post-intoxication incapacitation.The mechanism of action of the currently available prophylaxis pyridostigmine is to protect a fraction of the enzyme from binding with irreversible OP AChE-inhibitors. However, because of its structure, pyridostigmine hardly penetrates the brain and will therefore not protect the brain AChE sufficiently. This may lead to brain damage and post-intoxication incapacitation. Therefore, alternatives for pyridostigmine should be evaluated. For the near future, prophylaxis with physostigmine and scopolamine seems to be the best choice and most promising alternative to prevent OP intoxication. For the intermediary term, research towards compounds with another mechanism of action, like procyclidine, should be considered. For the long term the use of scavengers can be evaluated. This report is a new reviewed version of the former report PML 2002-A94 'Profylaxis against Organophosphorus nerve agents – state of the art'.

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Prophylaxis, pre-treatment, therapy, organophosphate, nerve agents, intoxication, chemical weapons, protection	

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16 ex. TNO Defensie en Veiligheid, vestiging Rijswijk,
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17 ex. TNO Defensie en Veiligheid, vestiging Rijswijk,
Medewerker BU4/DT: H v. Helden

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